Effects of Long-Term Storage on the Stability of *Op*NPV DNA Contained in TM Biocontrol-1â

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Abstract

Orgyia pseudotsugata nucleopolyhedrovirus (*Op*NPV) DNA was extracted from samples representing 10 lots of TM Biocontrol-1® stored at -10°C for 5 to 15 years and digested with the restriction enzymes *Bgl* II, *Pst* I and *Sal* I. DNA from the *Op*NPV virus strain (MEM-75-STANDARD) used to produce the TM Biocontrol-1® lots was also extracted and digested. No restriction fragment length polymorphisms were observed in any of the samples, and there was no evidence of DNA degradation. This indicates that long-term cold storage of TM BioControl-1® had no adverse effect on the quality of the *Op*NPV DNA. In addition to the expected >23 kb *Op*NPV DNA, extracts from lots 4a, 5b and 6 contained 10 additional nucleic acid segments, ranging in size from 0.9 to 4.2 kb. The electrophoretic profile of these segments was characteristic of *Orgyia pseudotsugata* cytoplasmic polyhedrosis virus (*Op*CPV). RNase A/DNase I treatment showed that the nucleic acid contaminants were composed of RNA, suggesting that lots 4a, 5b and 6 contained *Op*CPV as well as *Op*NPV.

Key words: Orgyia pseudotsugata; nucleopolyhedrovirus; biological insecticide; TM Biocontrol-1; long-term storage; OpNPV DNA

1. Introduction

TM Biocontrol-1® is a biological insecticide that was produced and registered by the United States Department of Agriculture (USDA) Forest Service for the purpose of suppressing outbreaks of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough). TM Biocontrol-1® contains the active ingredient *Op*NPV, a naturally occurring nucleopolyhedrovirus effective in controlling *O. pseudotsugata* populations because it is highly pathogenic to *O. pseudotsugata* larvae (Martignoni, 1999; Hughes, 1976; Hughes and Addison, 1970).

To propagate *O. pseudotsugata* virus, host insects free of endogenous viral infections are required. An inbred colony of virus-free *O. pseudotsugata*, called the GL-1 strain, was established from eggs collected in 1965 near Goose Lake in northern California (Martignoni, 1999). This colony was maintained at the USDA Forest Service Pacific Northwest Research Station, Forestry Sciences Laboratory, in Corvallis, Oregon until 1994 (Hadfield and Magelssen, 1995), and at the Pacific Forestry Centre, Canadian Forest Service, Natural Resources Canada, in Victoria, B.C. since late 1994. From 1980 to 1992, *O. pseudotsugata* virus was propagated in late instar GL-1 larvae at the Corvallis Forestry Sciences Laboratory. The virus-killed larvae were frozen and sent to companies for processing to recover virus from the dead larvae and thus produce TM Biocontrol-1®. The first lot was packaged in 1985 and the last lot was packaged in January 1995 (Hadfield and Magelssen, 1995). The exclusive source of virus for all of the production lots of TM Biocontrol-1® was a strain of the multinucleocapsid *Op*NPV (OpMNPV) called MEM-75-STANDARD, which was established by the USDA Forest Service in 1978. The propagation and amplification of OpMNPV followed the “seed-lot system” used by Fox et al. (1943) for the production of a strain of yellow fever virus. This system was implemented to avoid contamination of the pathogen, to ensure consistency between lots, and to minimize the chance of mutation of the virus. No more than two passages of virus separated the produced lots from the primary “seed lot” of the MEM-75-STANDARD strain.

Production of TM Biocontrol-1® from infected *O. pseudotsugata* larvae required a method that concentrated the viral inclusion bodies and excluded most of the unwanted insect debris. Larval cadavers were homogenized in water and the slurry was centrifuged to concentrate the polyhedral inclusion bodies (PIBs). The virus-rich sediments were lyophilized, screened to a fine powder, and vacuum-packaged for long-term storage (Martignoni, 1999). Although the USDA Forest Service could rear large numbers of infected larvae, they did not possess the equipment necessary to process large quantities of the final TM Biocontrol-1® product. In 1983, the USDA-Agricultural Research Service processed the first batch of TM Biocontrol-1® from 18 kg of infected larvae. A portion of this batch was used for research, and the remainder, 1680 acre-doses (representing lot 1), was returned to the USDA Forest Service. When the cooperative project was initiated in 1998, only 480 acre-doses of lot 1, which had been in cold storage the longest (since 1985), remained (Hadfield and Magelssen, 1995).

Large scale processing of TM Biocontrol-1® was subsequently contracted out to three different companies (Table 1). The first contract awarded by the USDA Forest Service was to Reuter Laboratories Inc. in 1986, and this company processed 147,600 acre-doses (lots 2, 3, 4 and 5). The next contract was awarded to Espro Inc. in 1988, and this company processed 170,566 acre-doses (lots 6 and 7). In 1990 Espro Inc. was acquired by Crop Genetics Inc., and this company continued the processing under contract for the USDA Forest Service. Crop Genetics produced 108,784 acre-doses (lots 8, 9 and 10). Virus production ceased in 1992 after an adequate supply of TM Biocontrol-1® had been produced and stored (Hadfield and Magelssen, 1995).

The purpose of this investigation was to examine the stability of the *Op*NPV genomic structure contained within the stored TM Biocontrol-1® samples.

2. Materials and Methods

2.1. Extraction of OpNPV and OpCPV from TM Biocontrol-1â

Seventeen samples of TM Biocontrol-1® obtained from the USDA Forest Service for an efficacy study (Kukan et al., 2001) were used as a source of *Op*NPV DNA. These samples represented the different package sizes (one to three) produced for each of the 10 lots of TM Biocontrol-1® processed and stored from 1985 through 1995 (Table 1), with the exception of one package (8a) that contained less than 700 total acre-doses. The four lots and 13 sub-lots examined in this study (Table 1) cover the entire range of time that TM Biocontrol-1® was held in cold storage (5 to 15 years).

To extract *Op*NPV DNA, 0.5-1.0 g of each TM Biocontrol-1® sample was suspended in 20 ml of sterile distilled water. The suspension was centrifuged at 2000*g* for 10 min. The supernatant was removed and the pellet resuspended in 20 ml of sterile distilled water, centrifuged at 1300*g* for 10 min, and the supernatant discarded. The upper white layer of the pellet was carefully suspended in 1.5 ml of sterile distilled water and transferred to a microfuge tube. Samples were centrifuged at 14,000*g* for 15 min to pellet the polyhedral inclusion bodies (PIBs). The pellet was washed three times with sterile TE (10 mM Tris-HCl pH 7.6; 1 mM EDTA, pH 8.0). The final pellet was resuspended in 0.1 M Na_2CO_3 and incubated at room temperature for 30 min to dissolve the polyhedra. Samples were centrifuged at 1000*g* for 5 min to pellet any large debris, and the supernatant, containing the virions, was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 30 min. The pellet was suspended in 500 µl of TES (100 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8; 0.1% SDS), 200 µg/ml proteinase K was added, and the samples were incubated overnight at 55^{o}C. Proteins were separated from viral DNA with two phenol: chloroform: isoamyl alcohol (25:24:1) extractions, and the DNA was further purified and concentrated using the GENECLEAN kit (Bio 101 Inc.). One µl of each sample was run on a

0.6% agarose gel containing 0.2% Synergel (Diversified Biotech) stained with 100 ng/ml ethidium bromide. Genomic *Op*NPV DNA was isolated from each production lot and digested (cut) with a series of restriction enzymes. The resulting *Op*NPV genomic profiles were compared to profiles of MEM-75-STANDARD DNA and *O. pseudotsugata* larval host DNA digested in the same manner. DNA concentrations were estimated by comparing the sample band intensities to those of 125 ng of Lambda DNA digested with *Hin*d III.

2.2. Extraction of OpNPV from infected O. pseudotsugata larvae

MEM-75-STANDARD was provided by the USDA Forest Service for comparison with the samples from the stored product. To obtain enough sample of MEM-75-STANDARD to use as a positive control, newly molted third instar *O. pseudotsugata* larvae (Goose Lake strain) were inoculated with a dilute sample of MEM-75-STANDARD (75-F-06). This single passage of the standard allowed for suitable amplification of the original form of the virus while at the same time minimizing the amount of valuable MEM-75-STANDARD used. The concentration of PIBs in MEM-75-F-06 was calculated using a hemocytometer and 5 µl aliquots of the stock solution. The number of PIBs was determined to be 1.63×10^7 PIBs/ml. Ten µl (16,300 PIBs) of a 1:10 dilution of the MEM 75-F-06 stock was applied evenly to the top of each artificial diet cube (6 mm $\times$ 6 mm $\times$ 3 mm) and allowed to soak in. The diet cube was placed in a 150 mm x 15 mm petri dish (Fisher Scientific) containing 10 fifth instar *O. pseudotsugata* larvae. When required, the cube was rehydrated with 10 µl of dH_20 to ensure that the larvae consumed as much of the virus contaminated diet cube as possible. Insects were reared until death, at which point the petri dishes with the cadavers were stored at –20^{o}C until required.

The stored frozen dead larvae were ground to a fine powder in a liquid nitrogen-cooled mortar and pestle with 0.5 ml STE-C buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0; 50 mM NaCl; 10 mM cysteine). The powder was transferred to microfuge tubes containing 1.0 ml of STE-C with 0.1% SDS and 0.06 mg/ml DNase I, and the tubes were incubated with gentle shaking at

room temperature for 20 min. The samples were centrifuged at 150*g* for 3 min to pellet insect debris. The supernatant was transferred to a fresh microfuge tube and centrifuged at 18,000*g* for 15 min to pellet the PIBs. The pellet was washed three times with TE buffer (pH 7.6), and the final pellet was resuspended in 500 µl of 0.1 M Na_2CO_3 and incubated with gentle shaking at room temperature for 30 min. The solution was centrifuged at 18,000*g* for 30 min, and the pellet was suspended in 500 µl of TES with 200 µg/ml proteinase K and incubated overnight at 55°C. Viral DNA was purified, concentrated, and its concentration estimated as described for the isolation of viral DNA from TM Biocontrol-1®.

2.3. Extraction of host DNA

O. pseudotsugata egg masses, free of *Op*NPV, were obtained from the laboratory colony of Goose Lake strain (GL-1) and reared on virus-free artificial diet (Thompson and Peterson, 1978) at the Pacific Forestry Centre since late 1994. Insects were reared until third instar, at which point they were removed from the growth chamber and stored at –20°C. These third instar larvae were used for the extraction of non-infected *O. pseudotsugata* DNA.

Frozen larvae were ground to a fine powder with a liquid nitrogen-cooled mortar and pestle. One ml of lysis buffer (10 mM Tris-HCl, pH 8.5; 5.0 mM EDTA; 0.2% SDS; 200 mM NaCl; 200 µg/ml proteinase K) was added to each powdered larva and the slurry incubated for several hours at 55°C. Following incubation, large debris were removed by a brief centrifugation at 150*g* for 3 min. DNA was purified from the supernatant using two phenol: chloroform: isoamyl alcohol (25:24:1) extractions. The DNA was further purified and concentrated using the GENECLEAN kit (Bio 101 Inc.), and the DNA concentration was estimated as described above.

2.4. Digestion of viral extracts and preparation of Southern blots

*Op*NPV DNA extracted from the samples of TM Biocontrol-1®, total genomic *O. pseudotsugata* DNA, and MEM 75-F-06 viral DNA were compared using restriction analysis. Twenty to 50 ng of DNA was digested with each of the restriction enzymes *Bgl* II, *Pst* I and *Sal* I, (NEB) overnight at 37°C. The digests were run in a 15cm x 20cm 0.6% agarose/1x TAE gel containing 0.2% synergel (diversified biotech) and 100 ng/ml ethidium bromide at 16mA for 15 h. Digested DNA was passively transferred to nylon membranes (Hybond-N^+) according to the manufacturer's protocol (Amersham Pharmacia Biotech).

2.5. Preparation of digoxigenin-labeled NPV probe

*Op*NPV genomic DNA was extracted from TM Biocontrol-1® as described above. One hundred ng of DNA was heat-denatured by incubation at 94°C for 10 min and immediately cooled on ice. To synthesize the DIG-labeled probe, denatured DNA was mixed with 151.6 µg/ml random hexanucleotides, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM DIG-dUTP. Two units of labeling grade Klenow (DNA Polymerase I) was added and the reaction mixture brought to a volume of 20 µl with sterile distilled water and incubated overnight at 37°C. Quantification of labeled probe was conducted according to the colourmetric detection protocol included in the DIG Labeling and Detection kit (Roche Applied Science).

2.6. Chemiluminescent detection of Southern blots

Southern blots were prehybridized for 2 hours at 65°C in standard hybridization buffer (5x SSC; 0.1% N-lauroyl-sarcosine; 0.02% SDS; 1% blocking reagent; Roche Applied Science). Digoxigenin-labeled genomic *Op*NPV DNA was denatured for 10 min in a boiling water bath, immediately cooled in an ice bath, and 10 ng/ml was added to fresh hybridization solution. Prehybridization solution was removed from the tubes and 20 ml of hybridization solution was

used for each 120 cm^2 blot. Blots were hybridized overnight at 65°C. Following hybridization, the solution containing the probe was transferred to a fresh 50 ml tube and stored at –20°C for use in subsequent hybridization reactions. The blots were washed twice at room temperature in 2x SSC, 0.1% SDS for 15 min each wash, then twice at room temperature in 0.1x SSC, 0.1% SDS for 30 min each wash. Detection of the digoxigenin-labeled DNA was conducted according to the manufacturer's protocol (Roche Applied Science) and visualized with exposure to Bio-Max X-ray film (Eastman Kodak, Inc.).

2.7. RNase A digestion of CPV samples

To confirm that the contaminant nucleic acids detected in lots 4a, 5b, and 6 were RNA, the DNA extracts from these samples were subjected to RNase A (Sigma) or DNase I (Sigma) digestion. Replicates of CPV-containing *Op*NPV DNA extract were digested with 1 unit/ml of RNase A, 1 unit/ml DNA or distilled H_2O for 20 min at 37°C. Digests were run through a 0.6% agarose/0.2% synergel gel containing 100 ng/ml ethidium bromide. Results were visualized by UV illumination and photographed.

3. Results

3.1. Physical properties of TM Biocontrol-1®

The various lots of TM Biocontrol-1® differed quite markedly in their physical characteristics. While these differences are not likely to affect the efficacy of the product, they were noted nonetheless.

Lot 1, produced by the USDA Forest Service, was very coarse. It appeared to be a rough mixture of relatively large insect debris intermixed with a more homogeneous coarse powder. This product was so coarse that it was necessary to grind it with a mortar and pestle in order to facilitate adequate sedimentation of the PIBs for extraction of the viral DNA.

The lots produced by Reuter Labs. Inc., lot numbers 2 through 5, appeared fairly homogeneous in texture. Large insect debris was not readily apparent, and the powdered component was finer than lot 1. Samples from lot numbers 2 through 5 did not require additional grinding with a mortar and pestle in order to extract viral PIBs, although additional grinding did aid in the recovery of PIBs.

Lot numbers 6 through 10, processed by Espro/Crop Genetics Inc., were very finely ground, and no large insect debris was apparent. Samples from these lots required no additional grinding with a mortar and pestle to aid in PIB recovery.

Extraction of viral DNA from the various samples of TM Biocontrol-1® yielded varying DNA concentrations, even though the same weight of sample was used for each extraction. Due to losses of DNA during the extraction and purification process, DNA recovery is not an effective way to quantify viral concentration in samples of TM Biocontrol-1®. However, in some cases, the differences in DNA recovered from lot to lot were larger than what could be reasonably be accounted for as experimental loss. This suggests that the quantity of PIBs (and virions) is not uniform from lot to lot.

3.2. Genomic variation in TM Biocontrol-1â

Each of the restriction enzymes *Bgl* II, *Pst* I and *Sal* I produced a characteristic restriction fragment length polymorphism (RFLP) profile. When DNA profiles from two or more individuals are different, they are said to contain polymorphisms (Bloom et al., 1996). In this study, polymorphisms were defined as a difference of at least one fragment in the RFLP profile of the sample DNA compared with that of the MEM-75 STANDARD DNA. However, no polymorphisms were detected in any of the 51 OpNPV DNA profiles produced using *Bgl* II, *Pst* I or *Sal* I to digest the 17 TM Biocontrol-1® samples.

For each restriction digest, the DNA fragment sizes were estimated by comparing the mobility of viral DNA fragments to the relative mobility of Lambda phage DNA digested with *Hin*d III.

3.2.1. Analysis of Bgl II RFLPs

Digestion of TM Biocontrol-1® samples with *Bgl* II produced nine observable fragments ranging in size from 1.0 to greater than 25.5 kb (Fig. 1). No polymorphisms were detected; all of the samples shared the same RFLP profile as the MEM positive control. The RFLP profiles in the TM Biocontrol-1® samples and the RFLP profile of the MEM-75-STANDARD DNA were consistent with previous findings in both size and number of fragments observed (Laitinen, 1995) (Table 2).

3.2.2. Analysis of Pst I RFLPs

Nineteen resolvable fragments, ranging from 1.0 to 6.5 kb, were identified in the TM Biocontrol-1® samples following *Pst* I digestion (Fig. 2). There were no observable polymorphisms in any of the TM Biocontrol-1® samples; all samples showed the same RFLP profile as that of the MEM-75-STANDARD. The *Pst* I profiles and estimated fragment sizes were consistent with results reported by Laitinen (1995), with the exception of a relatively faint 2.2 kb band noted in this study that was probably not observed by Laitinen (1995) (Table 3). The 2.2 kb band was also not clear in the TM Biocontrol-1® *Pst* I profile shown in Laitinen et al. (1996a), although the rest of this profile in her study matched the *Pst* I profiles produced in this study.

3.2.3. Analysis of Sal I RFLPs

Digestion of *Op*NPV DNA samples with *Sal* I produced the same electrophoretic profiles for all 17 samples and the control MEM DNA (Fig. 3). The *Sal* I profiles contained 18 fragments

ranging in size from 0.8 to 12.8 kb (Table 4). The number and molecular weights of these fragments were consistent with previously reported values (Laitinen, 1995). The *Sal* I profiles reported in this study also matched the TM Biocontrol-1® *Pst* I profile shown in Laitinen et al. (1996a) and closely corresponded to the *Sal* I profile of the *Op*NPV strain shown in Miller and Dawes (1978).

3.3. Presence of OpCPV in TM Biocontrol-1â

A large, >23 kb band of *Op*NPV DNA was observed in each successful DNA extraction from the TM Biocontrol-1® samples. In TM Biocontrol-1® samples from lots 4a, 5b and 6, smaller weight bands were also observed in addition to this large band. These additional bands did not represent polymorphisms in the *Op*NPV genome, because they were observed in the final DNA extract before the *Op*NPV genome was digested. Further examination of these TM Biocontrol-1® samples indicated that in each sample there were 10 bands present ranging in size from 0.9 to 4.2 kb (Fig. 4A). The band profile very closely matched the profile described for the double-stranded RNA virus, *Op*CPV (Laitinen, 1995; Laitinen et al., 1996b) (Table 5).

To test whether the smaller bands present in the TM Biocontrol-1® samples were indeed RNA, replicate samples from lot 4a extract were treated with DNase I, RNase A, or with distilled water (Fig. 4B). DNase I is an enzyme that specifically digests DNA while RNase A is an enzyme that specifically digests RNA. In the replicate treated with DNase I, the smaller bands remained intact while the *Op*NPV DNA was digested. Conversely, in the replicate treated with RNase A, the smaller bands had disappeared while the *Op*NPV genomic DNA remained intact. The replicate treated with distilled water showed both the smaller bands and the *Op*NPV genomic DNA. The fact that RNase A digested the smaller bands while DNase I did not indicates that these bands were composed of RNA.

The electrophoretic profile of the RNA segments in samples of TM Biocontrol-1® from lots 4a, 5b and 6 indicated that these lots were contaminated with *Op*CPV. It is not known when, where, or how the CPV contamination was introduced. However, because only 3 of the 17 TM Biocontrol-1® samples contained *Op*CPV, it is highly unlikely that the MEM-75-STANDARD used to inoculate the *O. pseudotsugata* larvae for the original production of TM Biocontrol-1® was the source of *Op*CPV. This is supported by the fact that the samples of MEM-75-STANDARD used as an experimental control in this study were also *Op*CPV free. The *Op*CPV can be considered a minor contaminant that may have been introduced inadvertently during production or processing of one lot and two sub-lots of TM Biocontrol-1®.

4. Discussion

*Op*NPV genomic DNA was successfully extracted from 17 samples, representing each of the one to three package size categories of the 10 lots of TM Biocontrol-1®. Viral DNA extracted from each lot was consistently observed as a single, high molecular weight band larger than 23 kb (data not shown). Digestion of *Op*NPV genomic DNA with each of the restriction enzymes *Bgl* II, *Sal* I and *Pst* I produced predictable and repeatable restriction fragments. All of the of TM Biocontrol-1® samples yielded RFLP profiles that were consistent with the control (MEM-75-STANDARD) as well as with the reported findings (Laitinen, 1995; Laitinen et al., 1996a; Miller and Dawes, 1978). These observations indicate that there has been no discernible degradation or mutation of the *Op*NPV DNA in any of the lots of the stored TM Biocontrol-1®.

Degradation of DNA is a random occurrence, and if the *Op*NPV DNA had been degraded, the DNA extracts would have appeared on a gel as a smear of unresolvable fragments. This was not the case, because in all of the samples *Op*NPV DNA was observed as a single large band prior to restriction digestion. Additionally, if DNA degradation had occurred,

it would have been apparent as either polymorphisms or smearing in the RFLP profiles of the samples. The DNA would have been digested as usual in areas where the DNA was still intact, but not in areas where the DNA had been degraded. The RFLP profiles of degraded samples would differ from the positive control (MEM-75 STANDARD) and from each other, since it is unlikely they would degrade at the same rate and in the same areas of the genome. Because no polymorphisms were detected in any of the stored TM Biocontrol-1® samples, it was concluded that long-term storage of the product has not affected the structure of the viral genome. This is not surprising, because the *Op*NPV virus is latent when stored at –10°C. With no chance to replicate, there is no opportunity for the genome to be altered by genetic mutation.

A small amount of contaminant was present in TM Biocontrol-1® samples from lot numbers 4a, 5b and 6. The electrophoretic profile and DNase I/RNase A treatments of this contaminant agreed with previous findings of Laitinen et al. (1996b) and Galinski et al. (1982), and confirmed the identity of the contaminant as *Op*CPV. This was unexpected, although the presence of *Op*CPV in *O.pseudotsugata* has been previously noted (Martignoni et al., 1969; Laitinen et al., 1996b). It is unlikely that the presence of *Op*CPV adversely affects the potency of the product. Cytoplasmic polyhedroviruses differ from nucleopolyhedroviruses in that they produce a chronic rather than lethal pathenogensis in their host (Payne and Martens, 1983). If anything, the presence of the *Op*CPV may enhance the pathogenicity of *Op*NPV, because the insects would have multiple infections to defend against rather than a single one. Synergistic effects have been reported between NPV and CPV (Tanada, 1956). However, further bioassays would be needed to confirm this assumption. CPV is a naturally occurring virus in field populations of O. pseudotsugata in some geographic locations (Galinski et al., 1994; Laitinen et al., 1996b; Williams et al., unpublished data).

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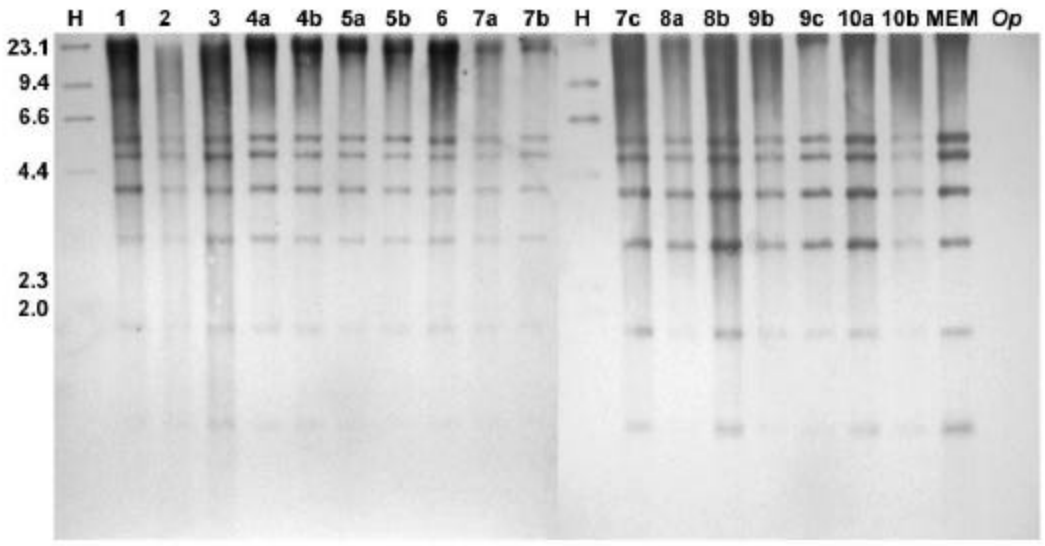
Fig. 1. RFLP profiles of 17 samples of stored TM Biocontrol-1® lots digested with *Bgl* II restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. *Op* indicates *O. pseudotsugata* host DNA. H denotes Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

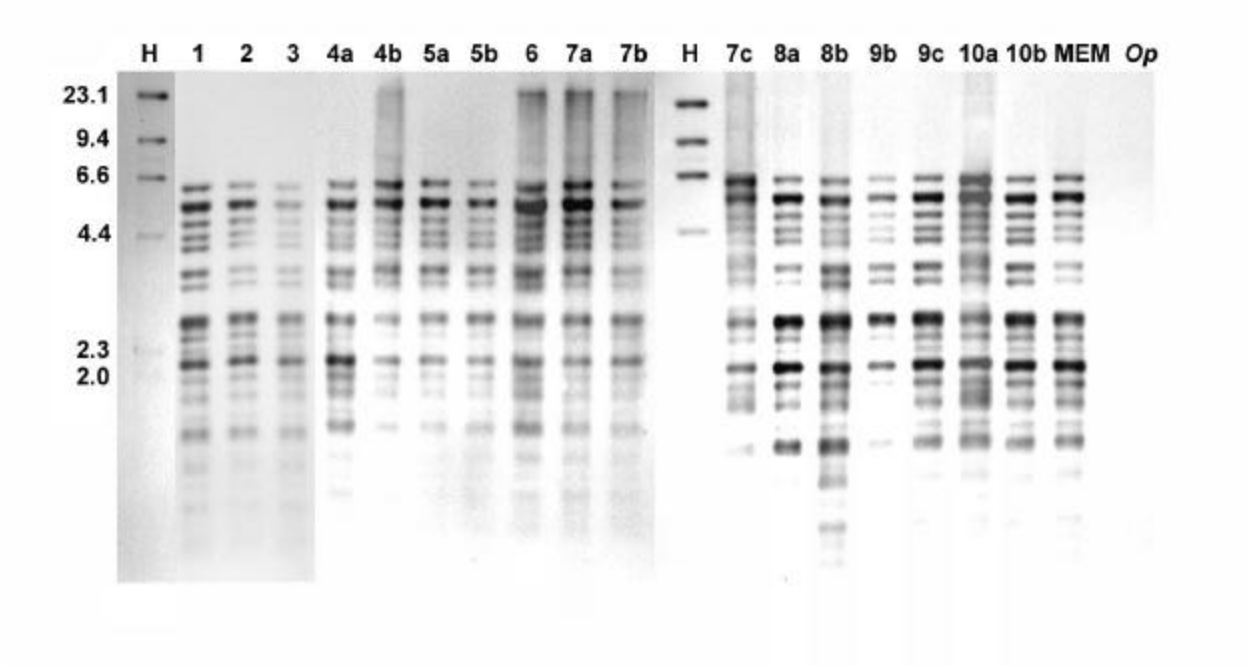
Fig. 2. RFLP profiles of 17 samples of stored TM Biocontrol-1® lots digested with *Pst* I restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. *Op* indicates *O. pseudotsugata* host DNA. H denotes Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

Fig. 3. RFLP profiles of 17 samples of stored TM Biocontrol-1® lots digested with *Sal* I restriction endonuclease. Values above lanes indicate lot number of the samples. MEM denotes MEM-75-STANDARD DNA. *Op* indicates *O. pseudotsugata* host DNA. H indicates Lambda *Hin*d III DNA standard. Numerical values on the left indicate the molecular sizes, in kilobases, of the Lambda *Hin*d III fragments.

Fig. 4. (a) *Op*NPV DNA extracts from samples of stored TM Biocontrol-1® from lot 4a (lane 2), lot 5b (lane 3) and lot 6 (lane 4) showing the presence of the contaminant, *Op*CPV. Numerical values on the left side indicate the molecular sizes of the Lambda *Hin*d III fragments (lane 1). Arrows on the right indicate the position of the 10 RNA segments of the *Op*CPV genome. (b) RNase A and DNase I digestions of lot 4a *Op*NPV DNA extract. Lane 1: Lambda *Hin*d III standards. Numerical values on the left indicate size in kilobases of the segments. Lane 2: *Op*NPV DNA extract incubated in distilled water. Both *Op*NPV and *Op*CPV genomes remained

intact. Lane 3: *Op*NPV DNA extract incubated with RNase A. Only the *Op*NPV genome remained intact. Lane 4: *Op*NPV DNA extract digested with DNase I. Only *Op*CPV genomic segments remained intact.


H
1
2
3
4a
4b
5a
5b
6
7a
7b
H
7c
8a
8b
9b
9c
10a
10b
MEM
Op
23.1
9.4
6.6
4.4
2.3
2.0


H
1
2
3
4a
4b
5a
5b
6
7a
7b
H
7c
8a
8b
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10a
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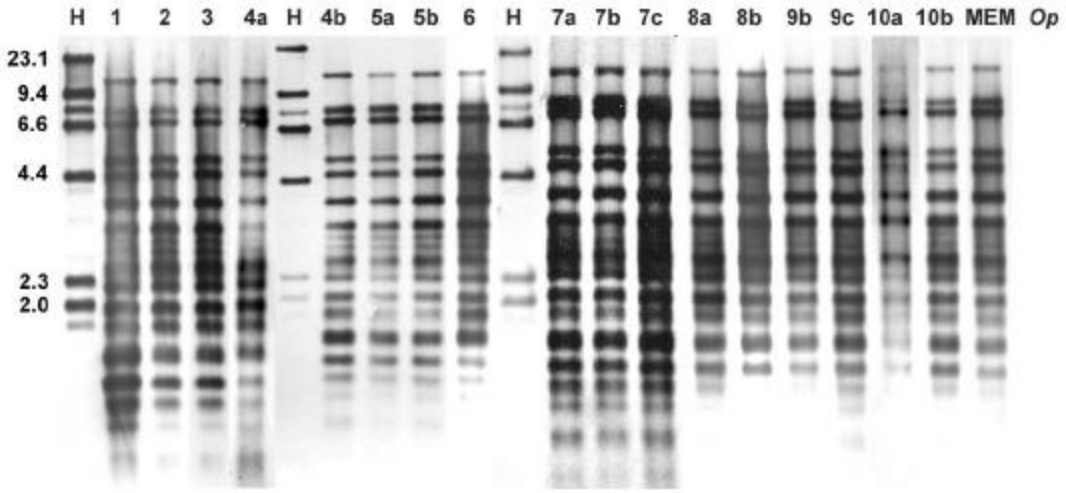
MEM
Op
23.1
9.4
6.6
4.4
2.3
2.0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1
2
3
4a
H
4b
5a
5b
6
H
7a
7b
7c
8a
8b
9b
9c
10a
10b
MEM
Op
23.1
9.4
6.6
4.4
2.3
2.0

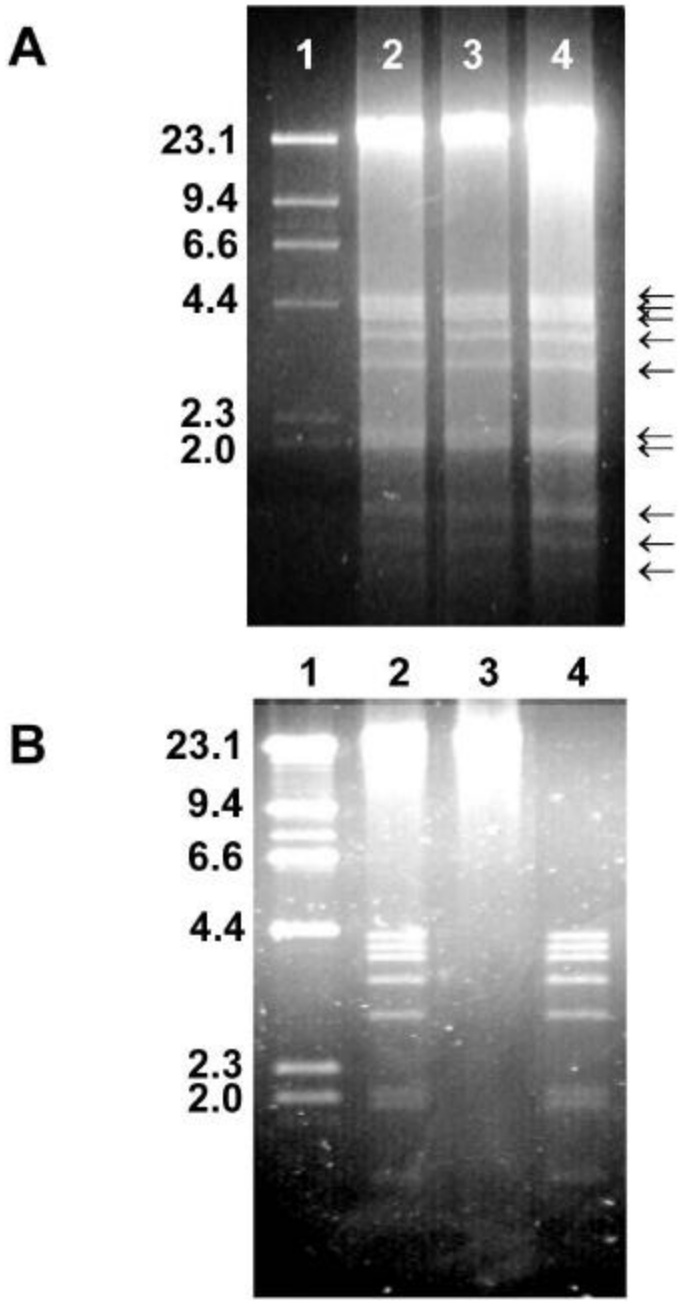
A
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
B
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0

Table 1. Amounts and storage dates of TM Biocontrol-1® processed from 1985 to 1992[a]

Lot #	Processing company	Amount (acre-doses)	Date stored
1	Agri. Res. Service	480	1985
2	Reuter Labs Inc.	18,000	1986
3	Reuter Labs Inc.	13,000	1986
4a	Reuter Labs Inc.	24,800	1986
4b	Reuter Labs Inc.	33,000	1986
5a	Reuter Labs Inc.	10,800	1986
5b	Reuter Labs Inc.	26,000	1986
6	Espro Inc.	17,383	1989
7a	Espro Inc.	48,300	1990
7b	Espro Inc	50,000	1990
7c	Espro Inc	50,000	1990
8a	Crop Genetics Inc.	690	1991
8b	Crop Genetics Inc.	68,000	1991
9b	Crop Genetics Inc.	5,784	1993
9c	Crop Genetics Inc.	9000	1993
10a	Crop Genetics Inc.	1,000	1995
10b	Crop Genetics Inc.	24,000	1995

[a] Information taken from Hadfield and Magelssen (1995).

Table 2. Estimated molecular weights, in kilobases, of *Op*NPV fragments generated by digestion with *Bgl* II restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	25.5	23.0+
2	24.3	23.0+
3	21.8	20.0
4	5.8	6.0
5	5.0	5.0
6	3.9	4.0
7	2.9	3.0
8	1.8	1.8
9	1.0	0.9

[a] Reported values taken from Laitinen (1995).

Table 3. Estimated molecular weights, in kilobases, of *Op*NPV fragments generated by digestion with *Pst* I restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	6.5	6.5
2	5.5	5.5
3	4.9	5.0
4	4.4	4.5
5	4.1	4.1
6	3.6	3.8
7	3.3	3.5
8	2.7	2.8
9	2.4	2.4
10	2.2	No band reported[b]
11	2.1	2.1
12	1.9	1.9
13	1.8	1.8
14	1.6	1.6
15	1.4	1.4
16	1.3	1.3
17	1.2	1.2
18	1.1	1.1
19	1.0	0.9

[a] Reported values taken from Laitinen (1995).

[b] The 2.2 kb band observed in this study is relatively weak and was probably not observed in the profiles produced by Laitinen (1995). This 2.2 kb band is also not apparent in the *Pst* I profiles shown in Laitinen et al. (1996a).

Table 4. Molecular weight estimates, in kilobases, of *Op*NPV fragments generated by digestion with *Sal* I restriction enzyme

Fragment	Estimated molecular weight	Reported molecular weight[a]
1	12.8	14.5
2	8.0	8.0
3	7.0	7.0
4	5.3	5.3
5	4.7	4.6
6	3.9	3.9
7	3.3	3.3
8	3.0	2.9
9	2.8	2.8
10	2.5	2.6
11	2.2	2.2
12	2.0	2.1
13	1.8	1.9
14	1.5	1.5
15	1.3	1.2
16	1.1	1.1
17	0.9	0.9
18	0.8	0.75

[a] Reported values taken from Laitinen (1995).

Table 5. Molecular weight estimates, in kilobases, for putative *Op*CPV found in TM Biocontrol-1®

Segment	Estimated molecular weight	Reported molecular weight[a]
1	4.2	4.3
2	4.0	4.1
3	3.8	3.6
4	3.4	3.4
5	2.9	2.9
6	2.0	2.0
7	1.9	1.95
8	1.3	1.3
9	1.1	1.2
10	0.9	1.0

[a] Reported values taken from Laitinen et al. (1996b).